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The Pineal Gland: A Neurochemical Transducer

Chemical signals from nerves regulate synthesis of melatonin and convey information about internal clocks.

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The pineal gland has become the subject of considerable investigation during the past decade because it provides a productive experimental model for studying circadian rhythms and regulation of end organs by nerves. In the mammal, the pineal gland rests between the two cerebral hemispheres and weighs about 100 milligrams in man and 1 mg in the rat (*1*). The pineal gland originates in the brain of the developing mammalian embryo, but it loses direct nerve connection with the brain soon after birth. The pineal parenchymal cells are innervated by sympathetic nerves (noradrenaline-containing) whose cell bodies lie in the superior cervical ganglia (*2*). Amphibian pineals have photoreceptive cells that can generate nerve impulses in direct response to environmental light (*3*). Photoreceptor elements, however, are not found in the mammalian pineal cells.

The beginning of the modern era in pineal research stemmed from the isolation and identification of the indoleamine melatonin (5-methoxy-*N*-acetyltryptamine) from bovine pineals by Lerner *et al.* (*4*). It then became possible to examine its localization, physiologic properties, formation, and metabolism. Melatonin is the most potent agent for causing contractions of melanophores in frog and fish skin. When treated with melatonin at concentrations of 10^{-13} gram per milliliter, the skin of many fish and amphibians rapidly blanches (*5*). The amphibian pineal contains melatonin and the enzymes that make it (*6*). These results indicate that melatonin causes changes in skin pigmentation in fish and amphibians when it is released from pineal organs. In the mammal, melatonin is synthesized mainly in the pineal (*1*), and it exerts inhibitory effects on gonads. When injected into birds, it causes a decrease in weight of the ovaries, testes, and oviduct (*1*). It delays vaginal opening and reduces ovary weight in young rats (*7*). When melatonin is implanted in the median eminence, the elevation in the content of leutinizing hormone (LH) in the pituitary following castration is blocked, and plasma LH concentration is lowered (*8*). Blinding of male hamsters causes a fall in the weight of testes, but when pineals are removed or when nerves to the pineal are cut the reduction in testicular weight is prevented. During proestrus in rats, melatonin inhibits ovulation by preventing the release of LH (*9*). The early morning elevation in plasma prolactin in male rats is mediated by increased release of a pineal hormone (*10*). In the sparrow, the pineal serves as a time-measuring system (*11*). The physiological aspects of the pineal have been reviewed recently (*12*).

Melatonin is synthesized almost exclusively within the pineal cell as follows (Fig. 1): tryptophan → 5-hydroxytryptophan → serotonin → *N* acetylserotonin → melatonin. Tryptophan is hydroxylated to 5-hydroxytrytophan by tryptophan hydroxylase (*13*). The latter amino acid is then decarboxylated by *l*-aromatic amino acid decarboxylase to form the biogenic amine serotonin. Serotonin then undergoes a complex fate. One portion is deaminated to 5-hydroxyindoleacetic acid by monoamine oxidase, and another portion leaves the pineal cell and is taken up by the sympathetic nerve terminal and stored together with the neurotransmitter noradrenaline (*1*) (Fig. 1). A third portion is acetylated to *N*-acetylserotonin by the enzyme serotonin *N*-acetyltransferase (*14*). This is a critical regulatory step, as will be shown later. *N*-Acetylserotonin is then *O*-methylated by hydroxyindole *O*-methyltransferase to form melatonin, *S*-adenosylmethionine serving as the methyl donor (*15*).

Hydroxyindole *O*-methyltransferase is highly localized in the pineal glands of mammals and birds. Small amounts of the enzyme are also present in the retina of the rat. In other classes (reptiles, amphibia, and fish), hydroxyindole *O*-methyltransferase is also found in the eye and brain as well as the pineal region (*16*). Although indirect, the evidence that the frog pineal blanches skin by secreting melatonin is compelling.

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Light and the Melatonin-Forming Enzyme

Rats exposed to constant illumination remain in persistent estrus. This condition could be reduced or prevented when rats were injected with extracts of bovine pineal glands (*17*). As a result of these findings Wurtman *et al.* (*17*) concluded that the pineal gland releases a substance that inhibits the gonads and that the formation and release of this substance is reduced when animals are kept in constant illumination. Fiske *et al.* (*18*) also found that pineal glands of rats exposed to continuous light weighed less. Then my colleagues and I (*7*) found that melatonin reduces the incidence of estrus in rats exposed to continuous light. It became apparent that environmental lighting might effect the melatonin-forming enzyme, hydroxyindole *O*-methyltransferase. Rats kept in continuous light for about 7 days showed a marked reduction in enzyme activity as compared to those kept in constant darkness (*19*). Thus, constant light decreased the activity of hydroxyindole *O*-methyltransferase, which in turn reduced the production of the gonad-inhibiting compound, melatonin. This reduction of melatonin synthesis in constant light would result in persistent estrus.

The question then arose as to how messages about environmental lighting could reach the pineal, which lies deep between the two cerebral hemispheres. The most likely possibility was a neural pathway. The mammalian pineal is heavily innervated by sympathetic nerve terminals, which are highly branched and contain swellings or varicosities that are in close juxtaposition with pineal parenchymal cells (Fig. 1). These varicosities contain numerous granulated vesicles that are the site of storage of the neurotransmitter noradrenaline (*20*). The nerve terminals that innervate the pineal can readily be destroyed by bilateral removal of the superior cervical ganglia. When rats with denervated pineals were kept in constant darkness or light, there was no longer a difference in hydroxyindole *O*-methyltransferase activity in the pineal (*21*). In blinded rats, continuous darkness or light had no effect on hydroxyindole *O*-methyltransferase activity, which suggests that the retina is necessary for transmission of light messages to the pineal. Bilateral lesions of the medial forebrain bundle, which contains noradrenergic and serotonergic nerves, also abolished the effects of environmental lighting on pineal hydroxyindole *O*-methyltransferase (*22*).

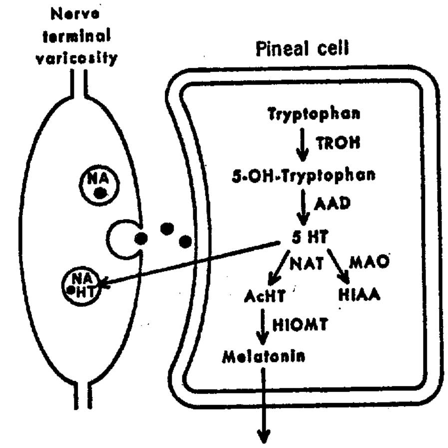

Fig. 1. The pineal cell, sympathetic nerve, and melatonin synthesis. Abbreviations: *TROH*, tryptophan hydroxylase; *AAD*, aromatic amino acid decarboxylase; *5 HT*, serotonin; *NAT*, serotonin *N*-acetyltransferase; *MAO*, monoamine oxidase; *AcHT*, *N*-acetylserotonin; *HIAA*, 5-hydroxyindoleacetic acid; *HIOMT*, hydroxyindole *O*-methyltransferase; and *NA*, noradrenaline.

In a series of experiments (*23*) it was shown that information about environmental lighting reaches the rat pineal as follows: retina → inferior accessory optic tract → medial forebrain bundle → medial terminal nucleus of the accessory optic system → preganglionic sympathetic tract in the spinal cord → superior cervical ganglia → postganglionic sympathetic fibers → parenchymal cells of the pineal.

Circadian Rhythms of the Pineal

Soon after melatonin was discovered, relatively large amounts of its precursor serotonin were found in the pineal (*24*); the serotonin was evenly distributed between the parenchymal cells and the sympathetic nerve terminals (*25*). Quay (*26*) then found a marked, 24-hour cycle in the serotonin content of the rat pineal. Peak levels of serotonin were reached at about midday (Fig. 2). Soon after nightfall, there was a rapid fall in serotonin content (*27*). The day-night rhythm of serotonin content in the pineal persisted unchanged in continuous darkness, but was abolished in rats that were kept in continuous light (*27*) (Fig. 2). Reversal of the lighting schedule (light kept on during the night and off during the daytime) changed the pineal serotonin rhythm in the pineal by 180° within 6 days (*28*). All of these experiments indicated that the daily rhythm in pineal serotonin is endogenous (circadian) but is synchronized by environmental lighting. Denervation of the pineal by removal of the superior cervical ganglia abolished the serotonin rhythm (*27*). Interruption of the nerve impulses from the central nervous system to the superior cervical ganglia and depletion of brain noradrenaline and serotonin with reserpine (*29*) also suppressed the pineal serotonin rhythm. These observations indicated that the circadian rhythm of serotonin is generated by sympathetic nerve terminals innervating the pineal, presumably by changes in the release of the neurotransmitter noradrenaline. The circadian serotonin rhythm appeared to be generated by a "clock" in the brain.

The circadian rhythms in serotonin content in the rat pineal appear as early as 6 days after birth (*30*). When lights were left on during the night, the nocturnal decline in serotonin content was prevented in adult and newborn animals. Lights left on during the night prevented the fall in serotonin in blinded 12-day-old rats. When the head of the 12-day-old rat was covered with a hood and the lights were on, the serotonin content fell at night. After the hooded rats were 27 days old, additional lighting no longer prevented the decline of serotonin at night (*30*). Thus, environmental lighting can reach the pineal gland by an extraretinal pathway in the newborn but not in the adult rat. Extraretinal pineal responses have also been found in birds (*11*).

There is a marked circadian rhythm in pineal *N*-acetyltransferase (*31*) (Fig. 2), which is 180° out of phase with that for serotonin. One hour after the onset of darkness, there is a 30- to 50-fold rise in the enzyme activity. A circadian rhythm in the melatonin content of the rat pineal has the same phasing as that of *N*-acetyltransferase. Like the serotonin rhythm, the *N*-acetyltransferase rhythm is abolished by denervating the sympathetic nerves to the pineal or by interrupting nerve impulses from the brain (*32*). Bilateral lesions in the suprachiasmatic nucleus (present in the hypothalamus) abolish the circadian rhythm of *N*-acetyltransferase in the pineal (*33*). This suggests that a biological clock in the brain sends fibers through, innervates, or is localized in the suprachiasmatic nucleus.

The observation that the circadian rhythms in pineal serotonin and *N*-acetyltransferase are abolished by cutting sympathetic innervation to the pineal indicated that there might be differences in the release of noradrenaline from these nerves during the day and at night. Brownstein and I (*34*) found a 24-hour rhythm in the turnover of noradrenaline in the sympathetic nerves innervating the pineal. More noradrenaline is utilized (presumably by release from nerve terminals onto the pineal cell) at night than during the day. This rhythm in turnover persisted in blinded rats and was abolished in continuous light. This strongly suggested that the circadian rhythm in the pineal cell is generated by diurnal release of the neurotransmitter noradrenaline. The circadian rhythm in sympathetic nerve activity is presumably driven by a biological clock arising in or transmitted via the suprachiasmatic nucleus in the hypothalamus.

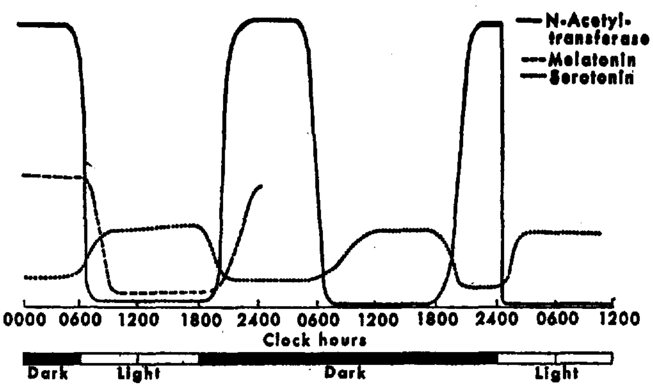

Fig. 2. Circadian rhythms in pineal *N*-acetyltransferase, serotonin, and melatonin.

Control of Pineal Indoleamine Metabolism in Organ Culture

The sympathetic nerves in the pineal contain both noradrenaline and serotonin, and it was not certain whether these compounds released from the nerve terminals exert their effects on pineal indoleamines. If, as appeared likely, the neurotransmitter noradrenaline stimulates the pineal cell, does it act on an α- or β-adrenergic receptor, and is adenylate cyclase involved? Also, which is the critical step in the synthesis of melatonin from tryptophan?

The rat pineal in organ culture proved to be a productive experimental tool to answer these questions. In such a system, the effects of biogenic amines, adrenergic blocking agents, and cyclic AMP (adenosine 3′,5′-monophosphate) could be examined directly, free of the complexities in vivo. In studies on pineal organ culture initiated independently by Shein and collaborators (*35*) and Klein *et al.* (*36*), [^{14}C]tryptophan was added to the culture media and the formation of radioactive serotonin, 5-hydroxyindoleacetic acid (the deaminated metabolite of serotonin), and melatonin was measured. The pathway of synthesis of melatonin in pineal organ culture was found to be the same as in the innervated pineal (Fig. 1). The addition of cycloheximide, an inhibitor of protein synthesis, to the incubation media completely inhibited the formation of [^{14}C]serotonin and [^{14}C]melatonin from [^{14}C]tryptophan, which indicates that synthesis of new enzyme protein was obligatory for the formation of melatonin (*37*).

The addition of *l*-noradrenaline to the pineal culture caused a marked increase in the formation of radioactive melatonin from tryptophan during 2 days of incubation (*35–37*). The stimulation of melatonin synthesis in the pineal organ culture by *l*-noradrenaline was prevented by the addition of *l*-propanolol, a β-adrenergic blocking agent (*37a*). α-Adrenergic blocking agents had no effect on the increased formation of melatonin in the presence of *l*-noradrenaline. When added to organ cultures, a variety of sympathomimetic amines such as *l*-adrenaline, dopamine, octopamine, and tyramine also stimulated the formation of melatonin from tryptophan (*37*). Serotonin or melatonin was without effect.

Many actions of noradrenaline are mediated by cyclic AMP. Evidence that noradrenaline might be acting via cyclic AMP in the pineal came from the observation that the catecholamine stimulates adenylate cyclase activity in homogenates of rat pineal (*38*). The addition of cyclic AMP to pineal organ culture, however, was without effect in stimulating melatonin synthesis. Dibutyryl cyclic AMP, a compound that, unlike cyclic AMP, is not metabolized by phosphodiesterase, markedly stimulated the formation of [^{14}C]melatonin from [^{14}C]tryptophan (*39*). These studies in organ culture indicated that noradrenaline released from sympathetic nerves increases melatonin synthesis by stimulating a β-adrenergic receptor on the membrane of the pineal cell. This stimulation results in activation of adenylate cyclase inside the cell to make cyclic AMP.

Klein and Berg (*40*) examined the effect of *l*-noradrenaline stimulation of hydroxyindole *O*-methyltransferase and *N*-acetyltransferase, the enzyme that converts serotonin to *N*-acetylserotonin. Noradrenaline caused only a small increase in hydroxyindole *O*-methyltransferase activity—not enough to account for the large increase in melatonin formation. On the other hand, the catecholamine caused about a 20-fold increase in *N*-acetyltransferase activity and melatonin formation after incubation of pineal organ culture for 18 hours. Dibutyryl cyclic AMP also markedly stimulated the *N*-acetyltransferase activity. The increase in *N*-acetyltransferase activity by either *l*-noradrenaline or dibutyryl cyclic AMP was blocked by the addition of protein synthesis inhibitors to the culture media. These observations suggested that the regulation of *N*-acetyltransferase synthesis by the β-adrenergic receptor is the critical step in the control of pineal melatonin synthesis.

Regulation of Pineal Circadian Rhythms

N-Acetyltransferase (*32*), serotonin (*26, 27*), *N*-acetylserotonin (*41*), and melatonin (*42*) undergo marked circadian rhythms (Fig. 2). In view of the diurnal rhythms in turnover of noradrenaline in sympathetic nerves innervating the rat pineal, it appeared likely that the circadian rhythms in the indoleamines and *N*-acetyltransferase are generated by day-night changes in the release of the neurotransmitter (*34*). Such a mechanism of regulation of pineal circadian rhythms was established by a series of experiments in vivo (*43*). After the onset of darkness at 1800 hours, *N*-acetyltransferase underwent a 30- to 50-fold increase in activity (Fig. 3). In the first hour after the lights were turned off at 1800 hours, only a small increase in enzyme activity was observed. Beginning at 1900 hours there was a sharp increase in enzyme activity, reaching a maximum at 2200 hours (Fig. 3). When lights were kept on after 1800 hours,

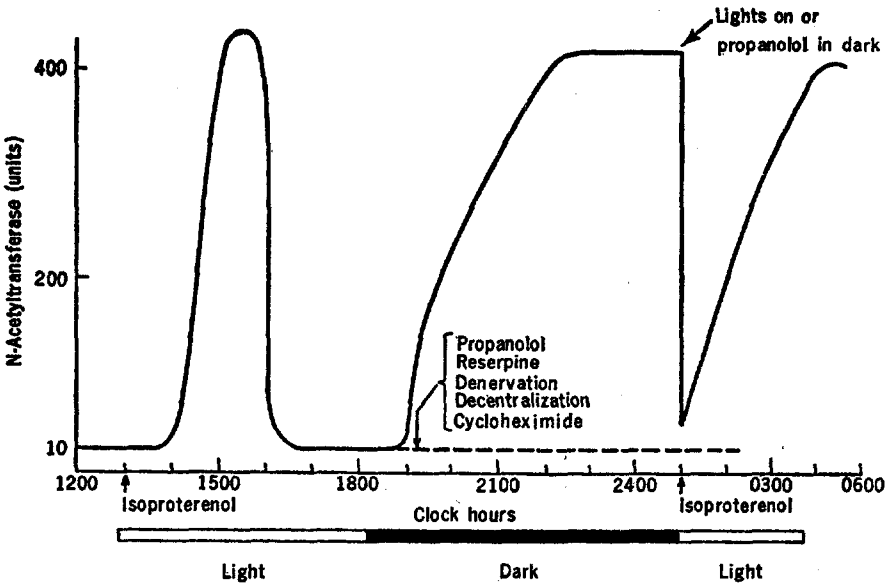

Fig. 3. Induction and suppression of serotonin N-acetyltransferase in the pineal.

however, there was no increase in N-acetyltransferase activity. Rats kept in darkness during the day from 1000 to 1600 hours also showed no daytime elevation in enzyme activity. These findings showed that both darkness and the proper setting of an internal clock are necessary for the nighttime increase in N-acetyltransferase activity. Interrupting sympathetic nerve impulses by bilateral removal of the superior cervical ganglia or preganglionic decentralization completely prevented the nocturnal elevation of N-acetyltransferase (*32*) (Fig. 3).

When *l*-propanolol, a β-adrenergic blocking agent, was administered to rats before the onset of darkness, the nighttime rise of enzyme was also blocked (*43*) (Fig. 3). Injection of an α-adrenergic blocking agent did not prevent the elevation. Reserpine, a drug that depletes nerves of both catecholamines and serotonin, also prevented the elevation of N-acetyltransferase. *p*-Chlorophenylalanine, a compound that depletes nerves of serotonin, had no effect on the circadian rhythm of N-acetyltransferase, which indicates that noradrenaline but not serontonin is involved in inducing the enzyme. Cycloheximide injected immediately before the onset of darkness blocked the rise of N-acetyltransferase, whereas actinomycin D, an inhibitor of RNA synthesis, had no such effect. These experiments clearly showed that noradrenaline released from sympathetic nerves stimulated the β-adrenergic receptor which in turn initiated events that lead to the synthesis of new N-acetyltransferase molecules.

Administration of the catecholamine *l*-isoproterenol during the daytime when both N-acetyltransferase activity and N-acetylserotonin content are low and serotonin content is high results in elevation of the enzyme (Fig. 3) (*44*) and its product N-acetylserotonin (*41*), and a fall of serotonin (*45*). The daytime rise in N-acetyltransferase and N-acetylserotonin and fall in serotonin induced by isoproterenol are blocked by prior administration of a β-adrenergic blocking agent. These observations indicate that circadian rhythms of pineal indoles are generated by changes in N-acetyltransferase activity which in turn are controlled by the β-adrenergic receptor. Thus, the rise in N-acetyltransferase at night causes a fall in serotonin and a rise in N-acetylserotonin, the precursor of melatonin (Fig. 2). The reverse occurs during the daytime. When rats are exposed to light during the night, there is a precipitous fall in pineal N-acetyltransferase (*43, 46*) (Figs. 2 and 3). Isoproterenol injected before the rats are exposed to light prevents the light-induced decrease in N-acetyltransferase activity (*43*). When rats are returned to darkness after 10 minutes of light, N-acetyltransferase activity begins to rise immediately and attains its initial level after 3 hours. Exposure to light during the night causes a rise in serotonin content as enzyme activity falls (*45*). Thus, maintenance of N-acetyltransferase activity requires continuous occupation of the β-adrenergic receptor. A brief exposure to light reduces or shuts off the release of nonadrenaline from sympathetic nerves, and a rapid fall in enzyme activity ensues (Fig. 3). The action of light on the retina is a stimulatory event, yet it inhibits the release of the neurotransmitter from sympathetic nerves in the pineal. This would indicate that, somewhere in the brain between the retina and the superior cervical ganglia, a stimulatory signal is converted to an inhibitory signal.

When the β-adrenergic blocking drug propanolol is injected into rats at night, N-acetyltransferase activity is decreased to less than 15 percent of its initial value within 10 minutes (*43*), a reduction similar to that caused by exposure to light (Fig. 3). Cycloheximide, given at night at a dose that immediately inhibits protein synthesis in vivo, results in a gradual decrease in N-acetyltransferase activity with a half-life of about 60 minutes (*43*). The rapid decrease in pineal N-acetyltransferase activity after light exposure or blockage of the β-adrenergic receptor (half-life of 5 minutes) and the slower fall in enzyme activity when protein synthesis is inhibited indicate at least two mechanisms for the degradation of N-acetyltransferase in vivo. The rapid decrease might result from the conversion of an active to an inactive form of the enzyme or from a disaggregation of subunits of the enzyme molecule. The slower decrease after inhibition of protein synthesis probably represents normal degradation of the enzyme.

The character of the N-acetyltransferase induction by catecholamines depends on the previous exposure to light. When rats are given isoproterenol after exposure to light for 6 hours, there is no increase in N-acetyltransferase for the first hour (Fig. 3). Then, between 1 and 3 hours after isoproterenol administration, enzyme activity rises, reaching a maximum after 2 hours (*43*) (Fig. 3). When rats are kept in darkness from 1800 hours to 2400 hours and then placed in light for 10 minutes, there is the expected rapid decrease in N-acetyltransferase activity. After a brief exposure to light following a prolonged period of darkness, isoproterenol injection causes an immediate increase in N-acetyltransferase activity without the 1-hour lag period (*43*) (Fig. 3). Cycloheximide, administered just before isoproterenol, blocks both the immediate and delayed increases in enzyme activity after isoproterenol injection. The delayed elevation of enzyme activity in the absence of β-adrenergic stimulation for long periods of time might indicate de novo synthesis of new enzyme molecules. The immediate elevation of N-acetyltransferase after a prolonged period of stimulation of the β-adrenergic receptor in darkness indicates an accumulation of messenger RNA or precursors necessary for the synthesis of N-acetyltransferase.

In pineal organ culture, noradrenaline causes a rapid increase in cyclic AMP (*47, 48*) as well as N-acetyltransferase and melatonin (*40*). Theophylline, a phosphodiesterase inhibitor enhances the effect of noradrenaline on pineal cyclic AMP, while β-adrenergic blocking agents prevent this effect. Thus noradrenaline stimulates N-acetyltransferase and melatonin production via β-adrenergic receptors linked to adenylate cyclase and cyclic AMP formation.

The temporal relationships between stimulation of the pineal β-adrenergic receptor, cyclic AMP content, and formation of N-acetyltransferase in vivo were reported by Deguchi (*49*). Injection of *l*-isoproterenol during the daytime (arrow A in Fig. 4) resulted in a 15-fold increase in pineal cyclic AMP content within 2 minutes which was maintained for 10 minutes; cyclic AMP content then fell to baseline levels 30 minutes after administration of the catecholamine. One hour after isoproterenol injection there was no change in N-acetyltransferase activity; during this time cyclic AMP content reached a peak and then returned to its initial level. Thirty minutes after cyclic AMP content returned to baseline, N-acetyltransferase activity rose, reaching a maximum 3 hours after injection of isoproterenol, and then returned to the low daytime value after 5 hours (Fig. 4). *l*-Propanolol injected before isoproterenol (arrow A in Fig. 4) prevented the rise of both cyclic AMP content and of N-acetyltransferase activity. When propanolol was injected 60 minutes after isoproterenol at a time when cyclic AMP content had already reached a peak and then returned to its initial level (arrow B in Fig. 4), the increase in N-acetyltransferase was still prevented. The administration of cycloheximide before isoproterenol (arrow A in Fig. 4) did not affect the rise and fall of cyclic AMP content, but it prevented the elevation of N-acetyltransferase activity. When the protein synthesis inhibitor was given after the rise and fall of cyclic AMP (arrow B in Fig. 4), the formation of N-acetyltransferase was still blocked. It can be concluded that stimulation of

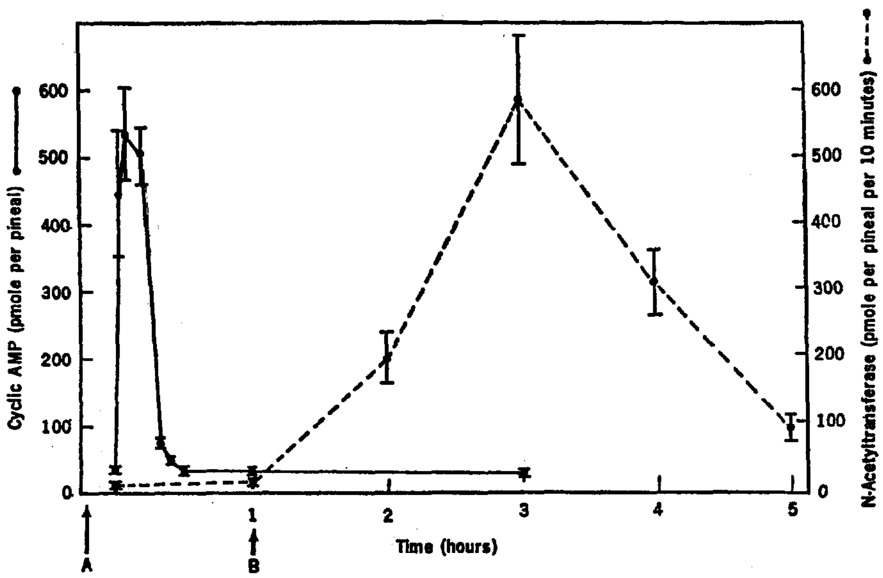

Fig. 4. Relation between cyclic AMP, N-acetyltransferase, and β-adrenergic receptors. *l*-Isoproterenol hydrochloride (5 mg per kilogram of body weight) was given intravenously to rats. Cyclic AMP and N-acetyltransferase activity was measured at times indicated. Arrows A and B are the times at which *l*-propanolol (20 mg/kg) was injected intravenously. Vertical bars indicate standard error of the mean. [From Deguchi (*49*), with permission of Academic Press]

the β-adrenergic receptor activates the formation of cyclic AMP via adenylate cyclase. This in turn induces the synthesis of new N-acetyltransferase molecules and thus more melatonin. Cyclic AMP may act on the translational process in protein synthesis, since cyclohexamide (but not actinomycin D) interferes with the induction of N-acetyltransferase. It also appears that there is an additional β-adrenergic receptor that is not involved in cyclic AMP formation but is concerned with the formation of N-acetyltransferase.

Supersensitivity and Subsensitivity in Pineal Response

An important and puzzling cellular phenomenon is the increase in responsiveness of the end organ after denervation, decentralization, or administration of drugs. Prior administration of cocaine or denervation of sympathetic nerves results in a markedly enhanced physiologic response to noradrenaline (*50*). This increased response is due mainly to a presynaptic event: the capacity of cocaine to block reuptake of noradrenaline into sympathetic nerves (*51*), a major mechanism of inactivation of the neurotransmitter (*52*). Denervation of sympathetic nerves also causes supersensitivity by destroying the catecholamine uptake mechanism and also by affecting the postsynaptic sites (*50*). Recent work has suggested a hypothesis for the changes in responsiveness of postsynaptic sites on the pineal cell after a variety of manipulations.

N-Acetyltransferase activity in the pineal is low during the daytime (*32, 43*). Various drugs and physiological manipulations were studied for their capacity to induce this enzyme during the daytime (*44*). Adrenaline, L-dopa, noradrenaline, and isoproterenol (Fig. 3) caused a marked increase in pineal N-acetyltransferase activity when injected during the daytime. The β-adrenergic blocking agent *l*-propanolol prevented the elevation of pineal N-acetyltransferase activity caused by the drugs or stress, while an α-adrenergic blocking agent did not.

To examine whether these compounds induced N-acetyltransferase directly or acted by the release of noradrenaline from the nerves innervating the pineal, the pineal was denervated by removal of the superior cervical ganglia or by chemical sympathectomy with 6-hydroxydopamine (*44*). When the pineal was denervated, administration of L-dopa increased N-acetyltransferase activity 100-fold as compared to 20- to 30-fold when the gland was innervated. Thus, denervation caused an increased responsiveness (supersensitivity) to catecholamines. Supersensitivity of denervated pineal with respect to N-acetyltransferase was also observed after administration of isoproterenol (*48*) or insulin or after stress

Table 1. Supersensitivity and subsensitivity in cultured pineals. Rats were denervated by bilateral removal of the superior cervical ganglia 7 days before they were killed. Isoproterenol-treated rats received the drug (2.0 mg/kg) 8, 16, and 24 hours before they were killed. Pineals were cultured for 10 hours with indicated concentrations of isoproterenol, and N-acetyltransferase activity was measured.

l-Isoproterenol (M)	N-Acetyltransferase (units)		
	Intact	Denervated	*l*-Isoproterenol-treated
1×10^{-9}	13 ± 2	$330 \pm 90^*$	
5×10^{-9}	330 ± 40	$1330 \pm 210^*$	$26 \pm 7^*$
2×10^{-8}	680 ± 160	$2190 \pm 330^*$	$70 \pm 24^*$
1×10^{-7}	940 ± 80	1180 ± 150	$320 \pm 50^*$
1×10^{-6}	1720 ± 180	1490 ± 170	1380 ± 110

* $P < .01$ compared to intact rats at the same isoproterenol concentration.

(*53*). The increased formation of N-acetyltransferase was prevented by β-adrenergic receptor blocking agents or by inhibitors of protein synthesis. These experiments demonstrated that stimulation of the pineal β-adrenergic receptors results in enhanced synthesis of the protein N-acetyltransferase (superinduction) after nerve denervation.

Superinduction of N-acetyltransferase appeared within 24 hours after removal of the ganglia innervating the pineal (*44*). It had been reported that pineal adenylate cyclase became more sensitive to noradrenaline, but not until 4 weeks after denervation (*54*). Thus, the appearance of adenylate cyclase supersensitivity does not explain the rapid appearance of superinduction of N-acetyltransferase. The β-adrenergic agonist *l*-isoproterenol was used to examine whether the changes in responsivity occurred on the postsynaptic membrane. This compound, unlike noradrenaline, is not taken up into sympathetic nerve terminals (*55*); any presynaptic effects are thus eliminated. Injection of small amounts of isoproterenol into rats resulted in tenfold greater induction of N-acetyltransferase in denervated pineals as compared to normally innervated pineals (*48*). When large doses of isoproterenol were injected, the same maximum induction was achieved in the intact and denervated pineals (*48, 56*).

Innervated and denervated pineals were then cultured in varying amounts of isoproterenol (*48, 56*). In the presence of the catecholamine, N-acetyltransferase activity increased gradually and reached a maximum after 6 to 10 hours in culture. Protein synthesis inhibitors or β-adrenergic blocking agents prevented this rise. Maximum induction of N-acetyltransferase in denervated pineals in organ culture occurred with an isoproterenol concentration of $5 \times 10^{-9}M$ (Table 1), while enzyme activity in the innervated pineal increased slightly at this concentration. Maximum induction in the innervated pineal was reached at an isoproterenol concentration of $1 \times 10^{-6}M$ (Table 1). Thus, the maximum response in the denervated pineal occurred at a dose of β-adrenergic agonist about 1 percent of that in the innervated gland. The maximum enzyme activity achieved, however, was the same in both cases.

To examine whether the increase in

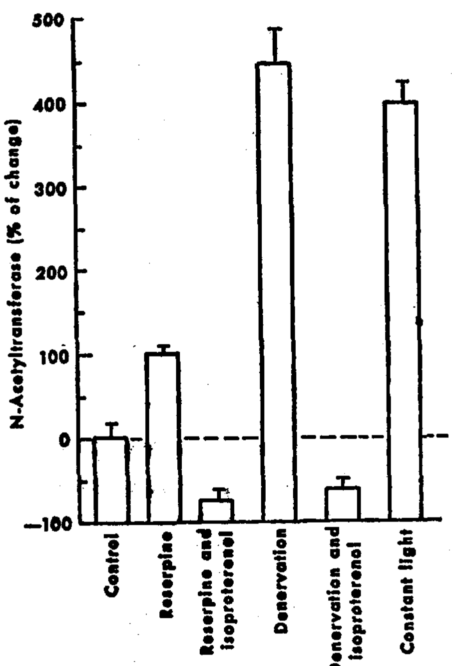

Fig. 5. Supersensitivity and subsensitivity of pineal N-acetyltransferase. Reserpine (1.5 mg/kg) was injected subcutaneously into rats 24 hours before they were killed. A group of reserpine-treated rats received *l*-isoproterenol (1.5 mg/kg) 8 and 16 hours before they were killed. Rats were denervated by bilateral removal of the superior cervical ganglia 48 hours before death. One group of denervated rats received *l*-isoproterenol (0.5 mg/kg) 8, 16, 24, and 32 hours before they were killed. One group of rats was exposed to light for 7 days.

sensitivity of the pineal response was due to absence of the neurotransmitter or to the nerve itself, rats were treated with reserpine to deplete the sympathetic nerve terminal of noradrenaline (*56*). Injection of a small amount of isoproterenol during the daytime resulted in a greater increase in pineal N-acetyltransferase activity in reserpine-treated rats as compared to nontreated animals (Fig. 5). Again, when higher doses of isoproterenol were injected, there was no difference in the maximum increase of N-acetyltransferase activity in normal and drug-treated animals. Pineal glands of rats with intact nerves that were depleted of noradrenaline by reserpine also showed a marked sensitivity in organ culture. The supersensitivity evoked by reserpine treatment developed less than 24 hours after the drug was given. Light reduces the release of noradrenaline from nerve terminals innervating the pineal. As predicted, cultured pineals of rats exposed to continuous lighting were many times more responsive to induction of N-acetyltransferase by isoproterenol than were pineals from rats under diurnal lighting conditions (*56*) (Fig. 5). Pineals were about ten times more responsive to catecholamines at the end of the light period (1800 hours) than at the end of the dark period, 0600 hours (*57*). The large (30-fold) elevation in pineal N-acetyltransferase at night could be explained by the reduced release of noradrenaline during the daytime causing supersensitivity to the increased neurotransmitter discharged with the onset of darkness. Exposure to long periods of light also resulted in increased elevation of pineal cyclic AMP content after noradrenaline injection (*47*).

These findings raised the possibility that supersensitivity after depletion of noradrenaline by reserpine or denervation might be prevented by the administration of the catecholamine. When isoproterenol was injected twice in a 24-hour period to denervated or reserpine-treated rats, not only was the superinduction of pineal N-acetyltransferase suppressed, but there was a markedly reduced induction of the enzyme when the pineal was later removed and exposed to isoproterenol in organ culture (*56*) (Fig. 5).

The induction of N-acetyltransferase in organ culture by low concentrations of isoproterenol was considerably reduced in the pineals of isoproterenol-treated rats (Table 1). However, the maximum response at high concentrations of the catecholamine was about the same in pineals in organ culture in both the isoproterenol-treated and untreated rats. Thus, continuous exposure of the β-adrenergic receptor to its agonist catecholamine results in a reduced responsiveness, or tolerance.

The effect of various doses of isoproterenol on stimulation of cyclic AMP in the pineal was examined 3 days after pineal denervation (*56*). Injection of small doses of isoproterenol resulted in more than twice the cyclic AMP increase in the denervated gland as in the innervated pineal. Large doses of isoproterenol gave the same maximum increase of cyclic AMP content in innervated and denervated pineals. Essentially the same cyclic AMP increases were obtained in innervated and denervated pineal glands in organ culture. There was no difference between the intact and denervated pineals with regard to induction of *N*-acetyltransferase activity when the organs were cultured in the presence of dibutyryl cyclic AMP (*56*), a compound that acts intracellularly. This indicated that denervation supersensitivity is due to changes proximal to the site of action of cyclic AMP, presumably on the β-adrenergic receptor on the outer cell membrane.

The increase in cyclic AMP in pineals from reserpine-treated rats was two to three times greater than in those from untreated rats (*56*). Treating rats with the protein synthesis inhibitor cycloheximide did not block the increased cyclic AMP response in reserpine-treated pineals. This experiment suggests but does not prove that new synthesis of receptor or of the adenylate cyclase system is not involved in the development of supersensitivity.

These experiments indicate that the responsiveness of the postsynaptic β-adrenergic receptor and the formation of the pineal hormone depend on previous exposure of the receptor to its neurotransmitter. When the noradrenaline discharged from sympathetic nerves is decreased or eliminated by denervation, decentralization, reserpine, or light, the responsiveness of the β-adrenergic receptor of the pineal to catecholamines and *N*-acetyltransferase induction and, ultimately, to melatonin formation is greatly increased (Fig. 6). The same magnitude of induction of *N*-acetyltransferase could be achieved with a much smaller amount of catecholamine in noradrenaline-depleted pineals as compared to pineals exposed to a complement of neurotransmitter normally discharged from sympathetic nerve terminals. If the number of catecholamine molecules impinging on the β-adrenergic receptor is increased, the pineal cell becomes less responsive to the catecholamine (Fig. 6). Thus, larger amounts of catecholamine are required to produce the same increase of *N*-acetyltransferase in these glands as compared to pineals receiving the normal amount of noradrenaline. This phenomenon is essentially the same as tolerance.

The shift in dose response in the induction of *N*-acetyltransferase by catecholamine in supersensitive and subsensitive pineals (Table 1) indicates a change in the affinity of the receptor for its agonist isoproterenol. Such changes in affinity suggest that the conformation of the β-adrenergic receptor has been changed by prior exposure to a larger or lesser amount

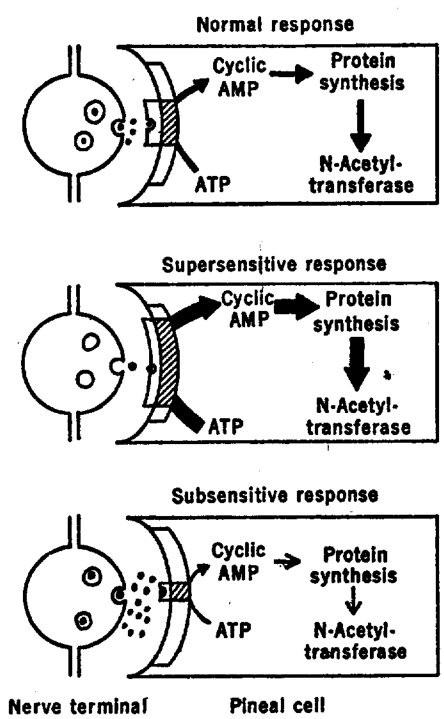

Fig. 6. Possible mechanisms for the development of supersensitivity and subsensitivity. After exposure to a reduced number of catecholamine molecules, there is an increase in the coupling of the β-adrenergic receptor on the outside of the pineal cell membrane to adenylate cyclase on the inner membrane. This leads to increases in the formation of cyclic AMP and synthesis of *N*-acetyltransferase when the β-adrenergic receptor interacts with catecholamines. The reverse occurs when the β-adrenergic receptor is exposed to excessive amounts of catecholamines; *ATP*, adenosine triphosphate.

of its agonist. It is also possible that receptor sites become more available when exposed to few agonist molecules or less available when exposed to an excess of molecules. Changes in sub- and supersensitivity can occur relatively rapidly, which makes changes in conformation or availability of the β-adrenergic receptor a likely possibility and a parsimonious adaptive mechanism for responsive cells.

Supersensitivity and tolerance to physiologic agents and drugs is not uncommon. Repeated administration of narcotic drugs results in rapid development of tolerance. On the basis of enzyme studies, it has been proposed that tolerance to opiates is caused by reduced availablity of receptor sites resulting from a continuous interaction of receptor with the narcotic drugs (*58*). In denervated muscle, an increase in the number of acetylcholine binding sites appears on the postsynaptic membrane (*59*), and the reverse occurs with exposure to large amounts of cholinergic agents (*60*). The number of insulin receptors decreases in fat cells of obese rats with high plasma insulin concentrations (*61*). Adenylate cyclase in the adrenal cortex is more responsive to adrenocorticotrophic hormone in rats in which the hormone has been previously depleted by hypophysectomy (*62*). In view of our findings with the pineal, changes in many physiologic and pharmacologic responses might be due to alterations in conformation or availability of receptor sites of responsive cells imposed by absence or excess of physiologic agents and drugs.

Summary

There are circadian rhythms in serotonin, serotonin *N*-acetyltransferase, *N*-acetylserotonin, and melatonin in the pineal which persist in continuous darkness and are abruptly abolished by exposure to light. These rhythms are generated by diurnal changes in the release of the neurotransmitter noradrenaline from sympathetic nerve terminals innervating the pineal. An increased discharge of noradrenaline at night stimulates the β-adrenergic receptor, which causes increased synthesis of serotonin *N*-acetyltransferase molecules inside the cell by mediation of an adenylate cyclase system. As the activity of *N*-acetyltransferase rises during the night, the concentration of

its substrate, serotonin, falls, and that of the product, *N*-acetylserotonin, rises. Increased synthesis of the pineal hormone melatonin then follows as a result of *O*-methylation of *N*-acetylserotonin by hydroxyindole *O*-methyltransferase. The responsiveness of the pineal β-adrenergic receptor and the consequent synthesis of *N*-acetyltransferase change; the receptor becomes supersensitive after decreased exposure to the catecholamines noradrenaline and isoproterenol and subsensitive after increased exposure to the catecholamines. The circadian rhythm in pineal amines appears to arise from a biological clock present in or near the suprachiasmatic nucleus in the hypothalamus. This clock in turn is modulated by inhibition by environmental light.

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